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RESEARCH ARTICLE

Detection and phylogenetic analysis of the membrane-bound nitrate reductase (Nar) in pure cultures and microbial communities from deep-sea hydrothermal vents

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Abstract

Over the past few years the relevance of nitrate respiration in microorganisms from deep-sea hydrothermal vents has become evident. In this study, we surveyed the membrane-bound nitrate reductase (Nar) encoding gene in three different deep-sea vent microbial communities from the East Pacific Rise and the Mid-Atlantic Ridge. Additionally, we tested pure cultures of vent strains for their ability to reduce nitrate and for the presence of the NarG-encoding gene in their genomes. By using the *narG* gene as a diagnostic marker for nitrate-reducing bacteria, we showed that nitrate reductases related to *Gammaproteobacteria* of the genus *Marinobacter* were numerically prevalent in the clone libraries derived from a black smoker and a diffuse flow vent. In contrast, NarG sequences retrieved from a community of filamentous bacteria located about 50 cm above a diffuse flow vent revealed the presence of a yet to be identified group of enzymes. 16S rRNA gene-inferred community compositions, in accordance with previous studies, showed a shift from *Alpha-* and *Gammaproteobacteria* to *Epsilonproteobacteria* as the vent fluids become warmer and more reducing. Based on these findings, we argue that Nar-catalyzed nitrate reduction is likely relevant in temperate and less reducing environments where *Alpha-* and *Gammaproteobacteria* are more abundant and where nitrate concentrations reflect that of background deep seawater.

Introduction

The biological cycling of nitrogen in the world's oceans involves to a great extent the inorganic nitrogen forms of ammonium and nitrate (Madigan *et al.*, 2000; Falkowski *et al.*, 2008). Several of the key reduction-oxidation (redox) reactions involving nitrate and ammonium cycling are carried out in nature almost exclusively by microorganisms; thus, microbial involvement in the ocean's nitrogen cycle is of great importance (Madigan *et al.*, 2000). Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) are two such key microbiological redox reactions involved in cycling many oxidation states of nitrogen. Although denitrification and DNRA result in different end products (dinitrogen gas and ammonium, respectively), the first step of nitrate reduction is identical and is carried out by the enzyme nitrate reductase (Argandona *et al.*, 2006). Currently, two types of

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respiratory and/or dissimilatory nitrate reductases, with different cell locations and structural/catalytic properties, have been described in bacteria: the membrane-bound (Nar) and the periplasmic (Nap) enzymes (Grove *et al.*, 1996; Philippot, 2005). Work on *Escherichia coli* and *Salmonella enterica*, which encode for both enzymes, has revealed important differences in conditions associated to the expression of these redundant enzymes. In these bacteria, the energy-efficient but low-affinity Nar enzyme is preferentially expressed under nitrate-rich conditions. In contrast, expression of Nap in these bacteria is favored under nitrate-limited conditions, as this enzyme provides a higher affinity but less efficient system, which, because of its location in the periplasmic space, does not require transport of nitrate into the cytoplasm (Potter *et al.*, 1999, 2001; Wang *et al.*, 1999; Rowley *et al.*, 2012). However, we do not know how the structural and catalytic differences of these enzymes determine their distribution in microorganisms or how they relate to different environments.

Several lines of investigation indicate that dissimilatory nitrate reduction is widespread in both high and moderate temperature deep-sea hydrothermal vent microorganisms (Götz *et al.*, 2002; Inagaki *et al.*, 2003, 2004; Vetriani *et al.*, 2004; Nakagawa *et al.*, 2005; Voordeckers *et al.*, 2005; Campbell *et al.*, 2006; Perez-Rodriguez *et al.*, 2010). The increasing concentration of nitrate with depth in the open ocean (Waksman *et al.*, 1933; Millero, 2006) suggests that nitrate-reducing microorganisms are important players in oceanic transformations of nitrate, and that nitrate reduction may be relevant also in the subsurface environments of mid-oceanic ridges, ridge flanks, island arc systems, abyssal plains, ocean island volcanoes, active continental margins and passive continental margins (Schrenk *et al.*, 2010; Xie *et al.*, 2011). However, while nitrate uptake has been investigated in the vent tubeworm, *Riftia pachyptila* (Girguis *et al.*, 2000; Robidart *et al.*, 2011), we are not aware of studies that assessed the diversity of nitrate-reducing bacteria at deep-sea hydrothermal vents.

Culture-independent assessments of the diversity of microbial communities in diffuse flow vents indicated that the majority of the microorganisms associated with the colonization of substrates exposed to sulfide-enriched fluids belonged to the *Epsilonproteobacteria* (Longnecker & Reysenbach, 2001; Lopez-Garcia *et al.*, 2003; Alain *et al.*, 2004). Furthermore, culture-dependent studies showed that most *Epsilonproteobacteria* isolated from deep-sea hydrothermal vents can reduce nitrate during anaerobic growth (reviewed in Sievert & Vetriani, 2012), and the genome sequences of several vent *Epsiloproteobacteria* indicated that these organisms encode for the periplasmic nitrate reductase, Nap and do not possess the membrane-bound enzyme, Nar (Nakagawa *et al.*, 2007; Campbell *et al.*, 2009; Sikorski *et al.*, 2010; Giovannelli *et al.*, 2011). Hence, we believe that Nap-mediated nitrate reduction by *Epsiloproteobacteria* may be important in sulfide-rich vent habitats at temperatures ranging between 20 and 60 °C. In a separate study, we investigated the distribution and diversity of the periplasmic nitrate reductase, Nap, in *Epsilonproteobacteria* and natural microbial communities from deep-sea hydrothermal vents.

In this study we investigated Nar-mediated nitrate reduction in both isolates and natural microbial communities from deep-sea hydrothermal vents. The membrane-bound Nar enzyme is composed of a complex, three-subunit quinol dehydrogenase that contains molybdenum bound to the bis-molybdopterin guanine dinucleotide cofactor at the active site. The *narG* gene, which encodes for the α subunit of the NarG enzyme, has been used previously as a functional marker to characterize nitrate-respiring bacterial communities in freshwater sediments and soils (Gregory *et al.*, 2000, 2003; Cheneby *et al.*, 2003; Alcantara-Hernandez *et al.*, 2009). These studies revealed that the Nar nitrate reductase is widespread in *Proteobacteria* (mostly *Alpha-* and *Gammaproteobacteria*) and *Firmicutes*, among other phyla.

Here, we have carried out the first comparative study of the 16S rRNA and the *narG* genes retrieved from vent natural microbial communities and from pure cultures obtained from sites on the Mid-Atlantic Ridge (MAR) and the East Pacific Rise (EPR). Our study provides insights into the distribution of nitrate-reducing bacteria encoding for the membrane-bound enzyme, Nar, at deep-sea hydrothermal vents.

Materials and methods

Sample collection

Three types of deep-sea hydrothermal vent habitats were sampled for the purpose of this study during two cruises aboard the R/V *Atlantis*: (1) Fragments of the wall of an active sulfide chimney venting fluids at 158 °C collected from the 'Rainbow' vent field on the MAR (36°14′ N, 33°54′ W; depth 2305 m), during cruise AT 05-03, *Alvin* dive 3678, July 2001. (2) Diffuse flow fluids from the East Wall vent field on the EPR (9°50′ N, 104°18′ W, depth 2500 m, fluid temperature: 25 °C) collected using titanium samplers and filtered shipboard on 0.2-μm Supor Gelman filters (Ann Arbor, MI) during cruise AT 15-26, *Alvin* dive 4109, May 2005. (3) White bacterial filaments samples collected from the mesh lid of an exclusion cage deployed for 1 year on a diffuse flow vent field on the EPR (Marker 89, EPR 9°50′ N, 104°18′ W, depth 2500 m, 2.5 °C) during cruise AT 15-26, *Alvin* dive 4102, May 2005. The lid of the exclusion cage was located

50 cm above the Marker 89 vent. All samples were collected using the manipulator of the DSV *Alvin* and stored in boxes on the submersible's working platform for the rest of the dive. On the surface, samples were transferred to the ship's laboratory and subsamples were frozen at −80 °C for nucleic acid extraction. *In situ* temperatures were measured using the probe on the DSV *Alvin* and sulfide measurements were conducted by *in situ* voltammetry as previously reported by Nees *et al.* (2008).

Reference strains

The reference strains used in this study were isolated from samples collected from deep-sea hydrothermal vents (predominantly from the EPR) in the course of several cruises (Table 1). Enrichment cultures were initiated shipboard under aerobic conditions in liquid media containing seawater salts and an organic carbon source (yeast extract/peptone, acetate or dodecane). Subsequently, pure cultures were isolated by three successive transfers of individual colonies on solid media. Identification of each isolate at the genus level was done by PCR amplification of the 16S rRNA gene from genomic DNA, sequencing, and phylogenetic analysis, as previously described (Crespo-Medina *et al.*, 2009). For the purpose of this study, all reference strains were grown aerobically in artificial seawater medium (ASW) with the following composition per liter: NaCl 24 g, KCl 0.7 g, $MgCl_2$ 7.0 g, yeast extract 3.0 g and peptone 2.5 g (Crespo-Medina *et al.*, 2009). To test for their ability to use nitrate as the terminal electron

Table 1. Characteristics of the reference strains used in this study

Genus (Accession No. for16S rRNA gene)	Growth in anaerobic ASW with NO_3^-	End product of nitrate respiration	Glucose fermentation	*narG* PCR product	Isolation location	Growth temperature (°C)
Alcanivorax sp. EPR7 (AY394866)	+	NO_2	+	+	Mk 119 Vent, East Pacific Rise	37–45
Alcanivorax sp. EPR8 (AY700225)	+	NO_2	+	+	Mk 119 Vent, East Pacific Rise	37–45
Alcanivorax sp. MAR12 (JQ764972)	+	NO_2	+	+	Broken Spur mussels, Mid-Atlantic Ridge	28
Klebsiella sp. EPRN2 (JQ764976)	+	NO_2	+	–	Bottom seawater, East Pacific Rise	30
Klebsiella sp. EPRN3 (JQ764975)	+	NO_2	+	–	Crab Spa vent, East Pacific Rise	30
Marinobacter sp. EPR35 (AY394884)	+	–	+	–	Mk 119 Plume, East Pacific Rise	28
Marinobacter sp. EPR49 (JQ764981)	+	NO_2	–	–	Mk 119 Plume, East Pacific Rise	28
Marinobacter sp. EPR59 (JQ764982)	+	N_2	+	–	Mk 119 Plume, East Pacific Rise	28
Marinobacter sp. EPR80 (JQ764983)	+	ND	ND	+	Tica Vent, East Pacific Rise	45
Marinobacter sp. EPR81 (JQ764969)	+	NO_2	–	+	Tica Vent, East Pacific Rise	46
Marinobacter sp. EPR108 (JQ764984)	+	ND	ND	+	Mk 89 Vent, East Pacific Rise	30
Marinobacter sp. EPR229 (JQ764973)	+	NO_2	–	+	Hydrocarbon–rich sediments, Guaymas Basin	35
Pseudomonas sp. EPR55	+	N_2	–	–	Mk 119 Plume, East Pacific Rise	28
Pseudomonas sp. P412-1 (JQ764978)	+	N_2	–	–	Palinuro Seamount, Mediterranean Sea	30
Pseudomonas sp. P412-2 (JQ764979)	+	N_2	–	–	Palinuro Seamount, Mediterranean Sea	30
Shewanella sp. EPRN1 (JQ764974)	+	NO_2	+	–	Bottom seawater, East Pacific Rise	30
Kribbella sp. EPR178 (JQ764977)	+	NO_2	+	+	*Alvinella* worm, East Pacific Rise	30

ND, experiment not done.

acceptor, all strains were also grown in anaerobic ASW medium supplemented with 14 mM KNO_3 (Table 1). Long-term stocks of the reference strains were prepared by adding 150 μL of sterile glycerol (Fisher Scientific) to 850 μL of liquid cultures grown overnight and were stored at −80 °C. Live cultures of the reference strains are available upon request.

The ability of representative reference strains to reduce nitrate to either nitrite or dinitrogen gas, and to ferment glucose, was determined using the API 20NE metabolic panels (bioMérieux, Durham, NC) following the manufacturer's instructions. Briefly, cells were grown in ASW medium for 24 h, harvested by centrifugation and suspended in 0.85% NaCl to a turbidity equivalent to 0.5 McFarland. The strips were then inoculated, incubated for 24 h at 29 °C (40 °C for *Alcanivorax* spp. EPR7 and EPR8) and the results recorded.

DNA extraction

Total genomic DNA was extracted from 2 g of chimney subsamples (Rainbow 3678) using the Ultra-Clean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) with the following protocol modifications: the bead beating step was extended for 20 s and it was followed by heating at 70 °C for 5 min. The UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories) was used to extract total genomic DNA from biomass collected on filters from about 2.4 L of hydrothermal fluids from East Wall, from *c.* 1 g of bacterial filaments from Marker 89 and from cells recovered from a 10-mL culture of each reference strain, following the manufacturer's protocol.

DNA amplification by polymerase chain reaction

A fragment of approximately 440 bp of the *narG* gene, encoding for the catalytic molybdenum-cofactor-containing subunit of the membrane-bound nitrate reductase (NarG), was amplified from the genomic DNA of the reference strains (Table 1) and from that extracted from the three natural communities. To this end, an initial polymerase chain reaction (PCR) reaction was performed with primers T37F and T39R (designed to amplify a fragment *c.* 1600 bp), followed by a nested PCR using primers T38R and W9F, according to Gregory *et al.* (2000). PCR conditions for amplification reactions were as follow: an initial denaturation for 5 min at 94 °C, followed by 30 cycles of 60 s at 94 °C, annealing at 55 °C for 60 s, and extension at 72 °C for 1.5 min, with an extension time of 10 min at 72 °C during the last cycle. Four independent PCR reactions per sample were carried out in parallel and pooled. Fragments of bacterial 16S rRNA genes ranging of 500–1400 bp were amplified by PCR and sequenced as described previously (Vetriani *et al.*, 1999, 2004).

Library construction, restriction fragment length polymorphism screening and sequencing

The amplified *narG* gene fragments from environmental samples were gel-purified using the Qiagen Gel Spin purification kit (Qiagen, Santa Clarita, CA), cloned into either pCR II or pCR4-TOPO plasmid vectors, and the ligation products were transformed into competent *E. coli* One Shot Top 10 competent cells (Invitrogen, Inc., Carlsbad, CA). Three 16S rRNA gene and three *narG* gene libraries were constructed for each of the natural microbial communities sampled: the EPR East Wall diffuse flow fluids library (Dive 4102), the EPR bacterial filaments at Marker 89 library (Dive 4109), and the MAR sulfide library from Rainbow chimney (Dive 3678).

Randomly chosen clones from each of the libraries (a total of 149 and 176 clones for 16SrRNA and *narG*, respectively) were analyzed for insert-containing plasmids by PCR followed by gel electrophoresis of the amplified products. The products were subjected to restriction fragment length polymorphism (RFLP) analysis using HaeIII and MspI restriction endonucleases for 16S rRNA genes and HaeIII for *narG* genes. Representative clones for each library showing unique RFLP patterns were selected and the sequences were determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). A summary showing the number of clones examined for each library is presented in Supporting Information, Tables S2–S6.

Phylogenetic analyses

The deduced amino acid sequences of NarG fragments (translated using the online tool EMBOSS Transeq, http://www.ebi.ac.uk/emboss/transeq/) were assembled using the AutoAssembler Program (Applied Biosystems), aligned using CLUSTALX v1.8 (Thompson *et al.*, 1997) and manually adjusted using SEAVIEW (Galtier *et al.*, 1996). Phylogenetic distances were calculated using the observed divergence matrix and the neighbor-joining method was used to evaluate tree topologies. GENEIOUS v5.4 was used to plot tree topologies (Drummond *et al.*, 2011) and their robustness was tested by bootstrap analysis with 500 re-samplings.

Gene richness and diversity analyses

The total number of clones identified by RFLP profiles were rarefied using ANALYTIC RAREFACTION v.2.0 (http://

www.huntmountainsoftware.com; Holland, 2008) to standardize for sample size. The 16S rRNA gene restriction profiles were rarefied to 59 clones for white filament- and diffuse fluids communities and to 37 for the black smoker gene clone libraries. The *narG* gene restriction profiles were rarefied to 55 clones for all three communities. Diversity indices for each of the 16S rRNA and the *narG* clonal profiles were estimated by calculating the Shannon–Wiener diversity index, H' (Shannon & Weaver, 1949):

$$H' = \Sigma - p_i \ln p_i$$

where p_i is the proportion of each restriction profile in the sampled library clones.

Nucleotide sequence accession numbers

The sequences from this study are available through GenBank under the following accession numbers: JQ608338–JQ608380 for the 16S rRNA gene sequences, JQ608381–JQ608453 for the *narG* gene sequences, AY394866, AY394884, AY700225, and JQ764968–JQ764984 for 16S rRNA gene sequences from the reference strains and JQ745299–JQ745303 for the *narG* gene sequences derived from the reference strains.

Results

Nitrate reduction and detection of the *narG* gene in reference strains

The reference strains used in this study were assessed for their ability to reduce nitrate and for the presence in their genomes of the NarG-encoding gene, *narG*.

Twenty-four strains of the genera *Alcanivorax* (four strains), *Marinobacter* (seven strains), *Klebsiella* (two strains), *Pseudomonas* (three strains), *Shewanella* (one strain), *Kribbella* (one strain), *Halomonas* (one strain), *Acinetobacter* (one strain), *Thioclava* (two strains), *Salipiger* (one strain), and *Rhodococcus* (one strain) were identified at the genus level by comparing their 16S rRNA gene to GenBank (Table 1 and Table S1).These strains were all aerobic mesophiles that grew at temperatures between 28 and 45 °C, and which were isolated from deep-sea hydrothermal vents located on the EPR, MAR, Guaymas Basin and the Mediterranean Sea (Table 1 and Table S1). Seventeen of these reference strains were able to grow under anaerobic conditions (in anaerobic ASW medium supplemented with nitrate) and belonged to the genera *Alcanivorax*, *Klebsiella*, *Marinobacter*, *Pseudomonas*, *Shewanella* and *Kribbella* (Table 1).

The gene encoding the catalytic α-subunit of the NarG, *narG*, was successfully amplified from *Alcanivorax* spp. strains MAR12, EPR7 and EPR8, *Marinobacter* spp. strains EPR80, 81, 108 and 229 and *Kribbella* sp. strain EPR178 (Table 1, Fig. 1). However, we only used five of them for phylogenetic analyses because we were unable to obtain a complete *narG* fragment product for *Kribella* sp. EPR178, *Marinobacter* sp. EPR108 or *Alcanivorax* sp. EPR7. We were unable to amplify the *narG* gene from *Marinobacter* sp. strains EPR49, EPR35 and from the *Pseudomonas*, *Klebsiella* and *Shewanella* strains (Table 1).

Fifteen of the 17 strains that were able to grow under nitrate-reducing conditions were subjected to metabolic and biochemical profiling using the API 20NE panels. Of the 15 strains profiled, including those whose *narG* gene could not be amplified, 14 were shown to reduce nitrate. The only exception was *Marinobacter* sp. strain EPR35, which did not reduce nitrate but fermented glucose. Of the nitrate-reducing strains, all *Pseudomonas* spp. and *Marinobacter* sp. EPR59 reduced nitrate to dinitrogen gas, while the remaining strains reduced nitrate to nitrite (Table 1). Furthermore, *Alcanivorax*, *Shewanella*, *Kribella* and *Klebsiella* spp., along with *Marinobacter* spp. EPR35 and EPR59, were shown to ferment glucose (Table 1).

Laboratory strains unable to grow in anaerobic ASW medium supplemented with nitrate, which also tested negative for the amplification of the *narG* gene, included members of the *Gammaproteobacteria* (*Alcanivorax* sp. strain EPR17, *Acinetobacter* sp. strain EPR111, *Halomonas* strain sp. EPR84), *Actinomycetes* (*Rhodococcus* sp. strain EPR110) and *Alphaproteobacteria* (*Salipiger* sp. strain EPR135, and *Thioclava* sp. strains EPR65 and EPR74 (Table S1).

16S rRNA gene-based community diversity

A total of 112 16S rRNA gene clones from the two EPR microbial communities, the filamentous bacteria (dive 4102) and the community associated with the East Wall diffuse flow fluids (dive 4109), were subjected to RFLP analyses, and representative clones were selected for sequencing (Table 2). A survey of 16S rRNA genes associated with the sulfide community of the Rainbow vent site on the MAR has been reported previously in Voordeckers *et al.* (2008).

The white filaments community (Dive 4102) was dominated by sequences related to cultured and uncultured *Alphaproteobacteria* (73%), while 25% belonged to cultured and uncultured *Gammaproteobacteria* and only 2% belonged to unknown epsilonproteobacterial species (Table 3). The majority of the 16S rRNA clones related to the *Alphaproteobacteria* clustered with uncultured and cultured *Sulfitobacter* spp. (52.5%), *Novosphingobium* spp. (37.5%), *Sphingomonas* spp. (5%) and *Erythrobacter* spp.

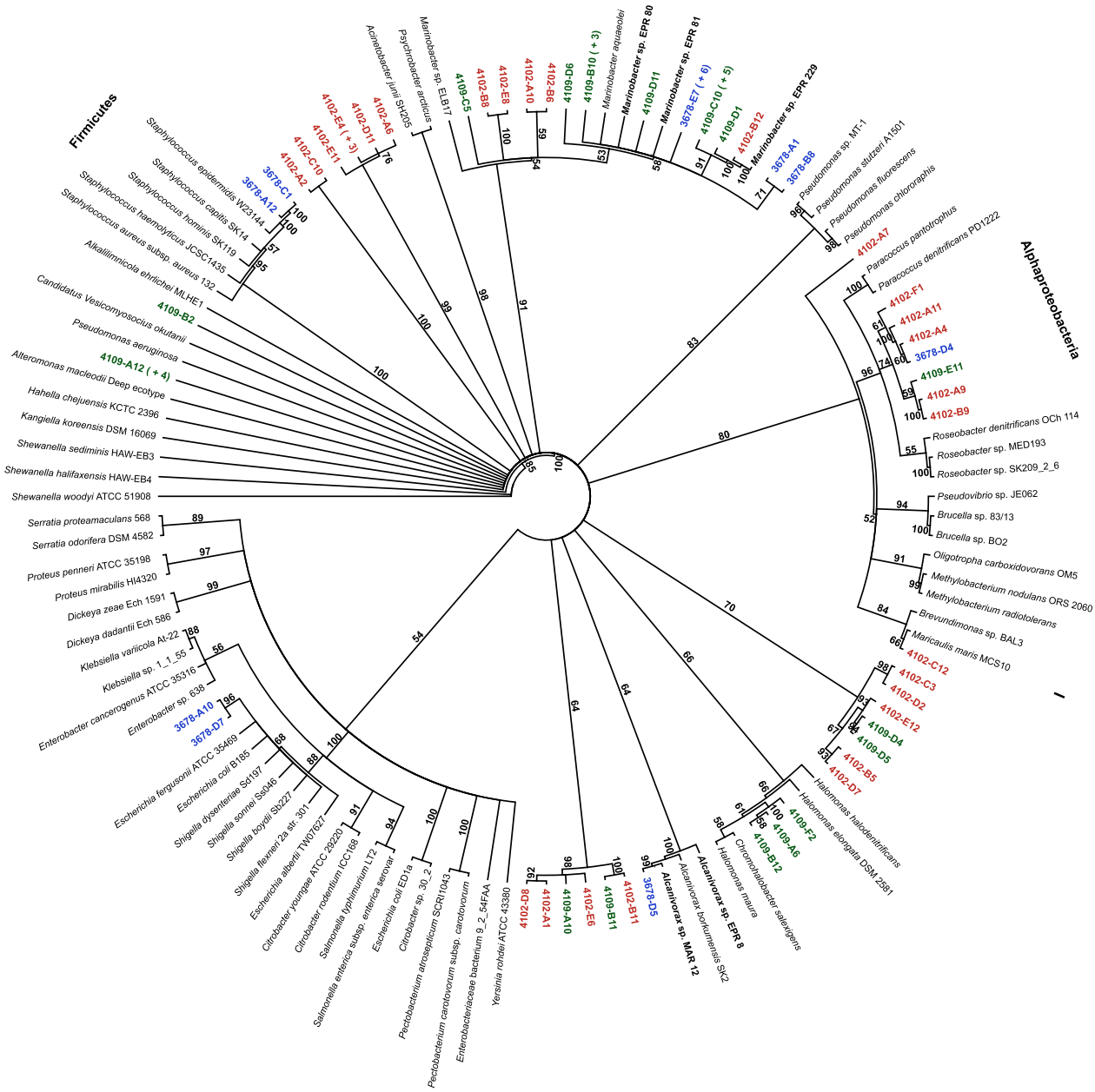

Fig. 1. Neighbor-joining phylogenetic tree inferred from nucleotide-deduced amino acid sequences of a fragment of the narG nitrate reductase, showing the position of reference strains and clones from vent natural communities. Bacterial filaments (4102; red); EPR East Wall diffuse flow (4109; green); MAR sulfide (3678; blue); reference strains from this study (bold/black). Bootstrap values based on 500 replications.

(5%). The gammaproteobacterial clones were related to *Pseudomonas* spp. (50%), uncultured spp. associated to polychaete annelid worms of the genus *Siboglinidae* (29%) and *Idiomarina* spp. (21%; Table 3).

The East Wall (Dive 4109) community was dominated by unknown phylotypes mostly related to uncultured and cultured *Epsilonproteobacteria* (56%). Of these *Epsiloproteobacteria*, 6% clustered with members of the genus *Sulfurovum*, while the others were related to uncultured *Epsilonproteobacteria* (47%) and to phylotype Dex80-90, an uncultured episymbiont of the tubeworm, *Alvinella pompejana* (47%; Table 3). The remaining clones were related to the *Gammaproteobacteria* (38.5%), *Spirochaetes* (3.5%) and uncultured *Bacteria* (1.75%; Table 3). Within the East Wall *Gammaproteobacteria*, the majority of the clones were represented by members of the *Halomonas* spp. (73%); *Marinobacter* spp. (9%), *Alishewanella* spp. (14%) and *Acinetobacter* spp. (4%; Table 3).

Table 2. Diversity of *narG* and 16S rRNA gene clone libraries

Gene	Sample type	Dive number	Sampling site	Unique RFLP profiles	Clones sequenced	Total clones screened	Shannon–Wiener index
narG	Bacterial filaments	Dive 4102	East Pacific Rise Marker 89	18	30	59	2.59
	Diffuse flow fluids	Dive 4109	East Pacific Rise-East Wall	21	28	60	2.56
	Black smoker	Dive 3678	Mid-Atlantic Ridge-Rainbow site	6	15	57	1.13
16S rRNA	Bacterial filaments	Dive 4102	East Pacific Rise Marker 89	11	16	55	2.35
	Diffuse flow fluids	Dive 4109	East Pacific Rise-East Wall	21	27	57	2.67
	Black smoker	Dive 3678	Mid-Atlantic Ridge-Rainbow site	11*	13*	37*	1.90

*Data from Voordeckers *et al.* (2008).

Table 3. Composition of the 16S rRNA gene clone libraries

Gene	Sample type (dive number)	Sampling site	% Unknown sequences; others	% *Alphaproteobacteria* genus (% of total)	% *Gammaproteobacteria* genus (% of total)	% *Epsilonproteobacteria* genus (% of total)
16S rRNA	Bacterial filaments (Dive 4102)	EPR, Marker 89	–	73 *Sulfitobacter* (52.5) *Novosphingobium* (37.5) *Sphingomonas* (5) *Erythrobacter* (5)	25 *Pseudomonas* (50) Uncultured (29) *Idiomarina* (21)	2 Uncultured
	Diffuse flow fluids (Dive 4109)	EPR, East Wall	2 Unknown 3.5 *Spirochetes*	–	38.5 *Halomonas* (73) *Marinobacter* (9) *Alishewanella* (14) *Acinetobacter* (4)	56 *Sulfurovum* (6) Uncultured (47) *A. pompejana* epysimbiont Dex80-90 (47)
	Black smoker* (Dive 3678)	MAR, Rainbow	3 δ-*Protebacteria*	–	–	97 *Caminibacter/Nautilia* (65) Uncultured (35)

*Data from Voordeckers *et al.* (2008).

A survey of 16S rRNA genes associated with the sulfide community of the Rainbow vent site on the MAR (dive 3678) showed that the majority of the clones were related to the *Epsilonproteobacteria* (97% of the total number of clones; 65% related to the Family *Nautiliales*; Table 3), as reported previously in Voordeckers *et al.* (2008).

Rarefied diversity estimates of the 16S rRNA clones, which minimize sample-size effects, showed that the black smoker community (dive 3768) had a lower richness than the filamentous bacteria (dive 4102) and the East Wall (dive 4109) communities (Fig. S1a). Details on the 16S rRNA-based diversity and richness of the three microbial communities are summarized in Table 2.

NarG-based community diversity

A total of 167 *narG* clones from the three microbial communities, the filamentous bacteria (dive 4102), the community associated with the East Wall diffuse flow fluids (dive 4109) and the community associated with the sulfide chimney at the Rainbow site on the MAR (dive 3678), were subjected to RFLP analyses. Seventy-three representative clones were selected based on their RFLP profiles, their sequences were obtained and identified as *narG* sequences. The deduced amino acid sequence of each clone was used to identify homologous proteins in the database and for phylogenetic analyses.

The NarG nitrate reductase from the filamentous bacteria (Dive 4102) were associated, for the most part, to proteins unrelated to any known organism (68%), while 22% and 10% of the proteins were related to *Alphaproteobacteria* and *Gammaproteobacteria*, respectively (Table 4). The alphaproteobacterial nitrate reductase clustered with sequences from *Paracoccus*/*Roseobacter* spp. (85%) and *Maricaulis* spp. (15%), while all of the gammaproteobacterial NarG retrieved from this community were related to *Marinobacter* spp., including several of the reference strains used in this study (e.g. *Marinobacter* sp. strains EPR 80, EPR 81 and EPR 229; Table 4, Fig. 1).

Table 4. Composition of the *narG* gene clone libraries

Gene	Sample type (dive number)	Sampling site	% unknown sequences	% *Alphaproteobacteria* genus (% of total)	% *Gammaproteobacteria* genus (% of total)	% *Firmicutes* genus (% of total)
narG	Bacterial filaments (Dive 4102)	EPR, Marker 89	68	22 *Paracoccus* (85) *Maricaulis* (15)	10 *Marinobacter* (100)	–
	Diffuse flow fluids (Dive 4109)	EPR, East Wall	27	10 *Paracoccus* (100)	63 *Marinobacter* (84) *Halomonas* (16)	–
	Black smoker (Dive 3678)	MAR, Rainbow	–	1.5 *Roseobacter* (100)	95 *Marinobacter* (89) *Escherichia* (9) *Alcanivorax* (2)	3.5 *Staphylococcus* (100)

The NarG associated with the East Wall diffuse flow fluids microbial communities (Dive 4109) were related to the nitrate reductase of *Gammaproteobacteria* (63%) and *Alphaproteobacteria* (10%), while 27% of the phylotypes were not related to any known organism (Table 4). Eighty-four percent of the gammaproteobacterial phylotypes were related to the nitrate reductase of *Marinobacter* spp., including several of the reference strains used in this study (Fig. 1), and 16% to *Halomonas* spp. All the East Wall alphaproteobacterial clones clustered with NarG sequences of *Paracoccus* spp.

The NarG nitrate reductase retrieved from the sulfide chimney collected at the Rainbow vent on the MAR (Dive 3678) were dominated by clones related to *Gammaproteobacteria* (95%); 3.5% and 1.5% of the sequences were related to the *Firmicutes* and to the *Alphaproteobacteria*, respectively (Table 4, Fig. 1). Eighty-nine percent of the gammaproteobacterial NarG clustered with *Marinobacter* spp., 9% with *Escherichia* spp. and 2% with *Alcanivorax* spp., including two of the reference strains used in this study, *Alcanivorax* sp. strains MAR 12 and EPR 8 (Fig. 1). Two of the remaining three clones retrieved from the sulfide community clustered with *Staphylococcus* spp. (*Firmicutes*) and one with the *Paracoccus*/*Roseobacter* group of the *Alphaproteobacteria* (Fig. 1).

Rarefaction curves for the *narG* clones showed that the black smoker community (dive 3678) had a much lower richness and diversity than the filamentous bacteria (dive 4102) and the East Wall (dive 4109) communities (Fig. S1b, Table 2). Overall, both surveys (16S rRNA and *narG* genes) indicated that the MAR black smoker community (dive 3678) was less rich and less diverse than the two EPR communities (Table 2).

Discussion

In this study we carried out a comparative analysis between the 16S rRNA gene and the gene encoding for the NarG from natural microbial communities collected from three different deep-sea hydrothermal vent habitats and from vent pure cultures.

Of all the reference strains able to reduce nitrate, we detected the *narG* gene in *Alcanivorax, Marinobacter* and *Kribbella* ssp., but we were not able to amplify the same gene from *Pseudomonas, Shewanella* or *Klebsiella* spp. (Table 1). Since the PCR primers used in this study were reported previously to amplify the *narG* gene from *Pseudomonas* spp. (Gregory *et al.*, 2000, 2003), it is possible that our *Pseudomonas* strains from the deep-sea carried a variant of the *narG* gene that is not a close match to our primers, or that these strains encoded for a different type of nitrate reductase. Moreover, with the exception of *Marinobacter* strains EPR80 and EPR108 (which grew in anaerobic ASW medium supplemented with nitrate and encoded for the *narG* gene), all the reference strains that grew anaerobically were also subjected to metabolic and biochemical profiling and were shown to reduce nitrate, either to nitrite or to dinitrogen gas (Table 1). The only exception was *Marinobacter* strain EPR35, which did not encode for the *narG* gene and, unlike the other *Marinobacter* spp., was unable to reduce nitrate (Table 1). Since the metabolic profile of strain EPR35 indicated that this isolate was able to ferment glucose, we can conclude that it grew by fermentation in anaerobic ASW medium. Within the genus *Marinobacter*, we found both fermentative (EPR35 and EPR59) and non-fermentative strains (EPR49 and EPR81; Table 1).

In the course of this study we detected several 16S rRNA gene and NarG sequences from the three natural communities that were genetically related to our reference strains (Tables 3 and 4, Fig. 1). The phylogenetic relatedness between environmental sequences and pure cultures of our vent reference strains whose physiology is known (e.g. ability, or lack thereof, to reduce nitrate) allows for a better interpretation of the environmental clones and underlines the importance of integrating gene surveys with studies of pure cultures. Although we did not find evidence for horizontal gene transfer (HGT) of the *narG*

gene in our reference strains (Fig. 1), occasional evidence for the lateral acquisition of this gene has been reported, e.g. in *Methylophaga* spp. (Auclair *et al.*, 2010). Therefore, the occurrence of HGT of the nitrate reductase-encoding gene in marine geothermal environments cannot be ruled out, and the validity of the *narG* gene as a taxonomic marker remains debatable (Philippot, 2002, 2005; Gregory *et al.*, 2003).

The samples used in this study represent three discrete deep-sea vent habitats characterized by different temperature and redox regimes. The EPR white filaments collected on the exclusion cage at Mk 89 on the EPR (Dive 4102) inhabited the coldest (*in situ* temperature 2.5 °C) and least reducing (H_2S concentration range: 0.2–32 μM; Nees *et al.*, 2008) habitat of the three. The 16S rRNA gene library derived from this community showed intermediate diversity among the three sites (*H′* index: 2.35; Table 2), with a prevalence of clones related to *Alpha-* and *Gammaproteobacteria* (73% and 25%, respectively; Table 3). It is likely that the low temperature, the distance from the hydrothermal fluid source, and the low sulfide concentration at which the white filament community developed could not support growth of sulfur-dependent *Epsilonproteobacteria.* Rather, background deep-ocean environmental conditions prevailed, as reflected by the dominance of *Pseudomonas, Idiomarina* and *Sulfitobacter*-related clones (Table 3). We found that 68% of the NarG sequences derived from this site were placed in four discrete clades unrelated to any known sequence (comprising clones 4102-E4, 4102-C10, 4102-C3 and 4102-E6, shown in Fig. 1, Table 4). The detection of these NarG sequences suggests that yet unrecognized bacteria encoding for the membrane-bound enzyme may play a role in nitrate reduction at background deep-ocean temperatures.

At the East Wall site (Dive 4109), fluids had an *in situ* temperature of 25 °C and a maximum free sulfide concentration of 175 μM (Nees *et al.*, 2008). The 16S rRNA gene library derived from the East Wall diffuse flow fluid was the most diverse (*H′* index: 2.67; Table 2), with the majority of clones related to *Epsilon-* (56%) and *Gammaproteobacteria* (38.5%; Table 3). We argue that this higher diversity reflects the heterogeneous nature of diffuse flow vents, which consist of a mixture of hydrothermal fluids and background seawater. Hence, East Wall fluids represent a microbial ecotone, where vent indigenous communities (i.e. the sulfide-dependent communities typically associated with vent fluids) mix with those associated with background seawater. On one hand, the moderate temperature and elevated sulfide concentration of the East Wall vent provide optimal conditions for mesophilic, sulfur-oxidizing *Epsilonproteobacteria*. These organisms are likely to be attached to the basalt or to the surface of invertebrates close to the venting source and are occasionally washed out by the fluid flux (Huber *et al.*, 2003; Alain *et al.*, 2004). In line with these observations, about half of the uncultured phylotypes at East Wall were related to epsilonproteobacterial phylotype Dex80-90, previously found in association with the tubes of *A. pompejana* (M.A. Cambon-Bonavita, V. Riou, K. Alain, V. Cueff, F. Lesongeur, G. Barbier and J. Querellou unpublished results). We also detected two clones (4109-A7 and 4109-E10) related to *Sulfurovum lithotrophicum*, a mesophilic *Epsilonproteobacterium* isolated from a deep-sea hydrothermal vent in the Mid-Okinawa Trough (Inagaki *et al.*, 2004). On the other hand, we also detected *Gammaproteobacteria* (38.5%) of the genera *Halomonas* and *Marinobacter*, among others, which are typically found in deep-sea pelagic environments, reflecting the entrainment of background seawater in the East Wall diffuse flow vent (Table 3; Kaye & Baross, 2000; Kaye *et al.*, 2011).

Phylogenetic analysis of the NarG from East Wall revealed a dominance of *Gammaproteobacteria*-like *narG* genes (63%), followed by previously unidentified clones as well as *Alphaproteobacteria*-related *narG* sequences (Table 4, Fig. 1). The overwhelming number of *Marinobacter*-related NarG detected in the East Wall community suggests that this gammaproteobacterial lineage may play a relevant role in nitrate reduction in this transition environment, where hydrothermal fluids mix with background seawater. Furthermore, phylogenetic analysis showed that several NarG environmental clones (e.g. 4109-B10, 4109-D11, 4109-C10, 3768-E7) recovered from the East Wall diffuse flow fluids (*c.* 50%; Fig. 1) were closely related to our *Marinobacter* reference strains isolated from hydrothermal vents (e.g. strains EPR 80, 81, 229; Fig. 1). Our data demonstrating that *Marinobacter* spp. reduce nitrate under the appropriate culture conditions and encode for the NarG (Table 1), along with our identification of a high proportion of *Marinobacter*-related NarG sequences in their native habitats, suggest that these organisms may play an active role in the reduction of nitrate in the transition zone where vent fluids mix with ambient seawater.

The MAR Rainbow black smoker (dive 3678) was the hottest (fluid temperature at the vent orifice 158 °C) and most reducing habitat of the three. The 16S rRNA and *narG* libraries from the Rainbow black smoker were the least diverse of all three habitats (*H′* indices: 1.90 and 1.13, respectively; Table 2). We attribute this to the steep temperature and redox gradients typical of black smokers, which restrict this habitat to specialized organisms (Karl, 1995; Lopez-Garcia *et al.*, 2003). The 16S rRNA gene library derived from the Rainbow black smoker was dominated by clones related to the *Epsilonproteobacteria* (97%), 65% of which belonged to the *Caminibacter*/*Nautilia* group, as previously reported by Voordeckers *et al.* (2008). This group of *Epsilonproteobacteria* includes thermophilic,

hydrogen-oxidizing and nitrate/sulfur-reducing organisms isolated from black smokers or *A. pompejana* tubeworms (Table 3; Alain *et al.*, 2002; Miroshnichenko *et al.*, 2004; Voordeckers *et al.*, 2005; Smith *et al.*, 2008; Alain *et al.*, 2009; Perez-Rodriguez *et al.*, 2010).

In contrast with the community composition inferred from the 16S rRNA gene analysis, our *narG* survey of the MAR Rainbow black smoker revealed that 95% of the sequences were related to *Gammaproteobacteria*, 89% of which clustered with the *Marinobacter* lineage (Table 4, Fig. 1). This discrepancy may be explained by the observation that *Epsilonproteobacteria* isolated from deep-sea hydrothermal vents encode for the periplasmic nitrate reductase, Nap, rather than for the membrane-bound enzyme, Nar (Nakagawa *et al.*, 2007; Campbell *et al.*, 2009; Giovannelli *et al.*, 2011). These findings suggest that, in the MAR Rainbow black smoker, we were able to capture the gammaproteobacterial fraction of the community that carried the membrane-bound enzyme. Because of the extent of our analysis, this rare fraction of the MAR microbial community was undetectable in our 16S rRNA-based survey (compare Tables 3 and 4). We hypothesize that, within the walls of the black smoker structure, *Epsilonproteobacteria* (including thermophilic organisms of the *Caminibacter/Nautilia* group) inhabit the more reducing, sulfidic microhabitats characterized by temperatures up to 60 °C, whereas *Gammaproteobacteria* inhabit the external, more oxidized zones of the structure, where the temperature is lower (Voordeckers *et al.*, 2008). Because nitrate is depleted in vent fluids but relatively abundant in deep seawater (about 40 μm in the Pacific Ocean; Millero, 2006), it is reasonable to expect it to diffuse inward from seawater into the porous chimney structure and become gradually depleted in the more reducing and hotter parts of the black smoker. According to this model, Nap-encoding *Epsilonproteobacteria* would inhabit regions of the black smoker where nitrate is more depleted, whereas Nar-carrying *Gammaproteobacteria* would be exposed to higher nitrate concentrations. This partitioning of the Nap/Nar enzymes in microorganisms along a nitrate gradient would be consistent with data showing that, under nitrate-rich conditions, the energy-efficient but low-affinity Nar enzyme is used. In contrast, under nitrate-poor conditions, the Nap enzyme may provide higher affinity but a less efficient system (Potter *et al.*, 1999, 2001; Wang *et al.*, 1999; Rowley *et al.*, 2012).

In conclusion, our study suggests that, at deep-sea hydrothermal vents, Nar-catalyzed nitrate reduction appears to be significant in temperate and less reducing environments, such as the white filaments in Mk 89 and East Wall diffuse flow fluids, where mesophilic *Alpha-* and *Gammaproteobacteria* are well represented both in the 16S rRNA and *narG* clonal libraries, and where the concentration of nitrate is closer to that of the background seawater. In particular, NarG-encoding *Gammaproteobacteria* related to *Marinobacter* spp. appear to occupy a niche where sulfidic-rich hydrothermal fluids mix with background seawater (East Wall fluids and Rainbow black smoker habitats). A yet to be identified group of NarG sequences was numerically dominant in the clone libraries derived from the white filament community. Future isolations of novel nitrate-reducing bacteria from the deep-ocean, along with their genome sequences and metagenomic/proteomic studies, may help to resolve the origin of these novel nitrate reductases, place them in a physiological context, and establish the significance of these organisms in the reduction of nitrate at low temperature and low sulfide vent sites.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Reference strains unable to grow in anaerobic ASW medium
Table S2. Data summary for 16S rRNA gene sequences recovered from the EPR white filament community (Dive 4102).
Table S3. Data summary for 16S rRNA gene sequences recovered from the EPR East Wall diffuse flow fluid community (Dive 4109).
Table S4. Data summary for *narG* sequences recovered from the MAR black smoker community (Dive 3678).
Table S5. Data summary for *narG* sequences recovered from the EPR filamentous bacteria community (Dive 4102).
Table S6. Data summary for *narG* sequences recovered from the EPR diffuse flow fluid community (Dive 4109).
Fig. S1. (A) Rarefaction curves of observed 16S rRNA gene libraries for sampling sites 4102 ($n = 59$), 4109 ($n = 59$) and 3678 ($n = 37$). The error bars are 95% confidence intervals. (B) Rarefaction curves of observed narG gene libraries for sampling sites 4102 ($n = 59$), 4109 ($n = 60$) and 3678 ($n = 57$). The error bars are 95% confidence intervals.